Exhibit 1

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Weiner *et al.*

Serial No.: 09/622,452 Group Art Unit: 1632

Filed: October 31, 2000 Examiner: Anne Marie Sabrina Wehbe

Title: VACCINES, IMMUNOTHERAPEUTICS AND METHODS FOR USING THE SAME

Declaration of Dr. David B. Weiner
Pursuant to 37 CFR § 1.132

I, David B. Weiner, do hereby declare:

1. I am a co-inventor of the subject matter claimed in the above-identified patent application.

2. I have performed and/or supervised the performance and/or collaborated in the performance of experiments designed to study the immunomodulatory effects associated with the use of DR5, and the nucleic acids that encode it, in DNA vaccines.

3. The attached manuscript, which is designated as Exhibit 1, contains data from experiments designed to study the immunomodulatory effects associated with the use of nucleic acid molecules that encode DR5 in DNA vaccines.

4. Experiments described in the manuscript include a comparison of immune responses induced in mice by injection of a plasmid that encodes an HIV antigen with those of immune responses induced in mice by injection of a combination of plasmid that encodes the HIV antigen and plasmid that encodes an Adjuvant, in which the Adjuvant was either DR5, OX40, Fas, RANK, or TNFR. Plasmid pVax, an empty vector plasmid, was used as a control Adjuvant.

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5. Data in the manuscript show mice receiving the combination of plasmid that encoded Antigen and plasmid that encoded DR5 exhibited enhanced immune responses compared to those observed in mice that received plasmid that encoded Antigen, or to those that received the combination of plasmid that encoded Antigen and plasmid that encoded one of the other Adjuvants.

6. Experiments described in the manuscript include a comparison of immune responses induced in mice by injection of a plasmid that encoded an HIV antigen only with the immune responses induced in mice by injection of a combination of plasmid that encoded an HIV antigen and plasmid that encoded DR5, and the immune responses induced in mice by injection of a combination of plasmid that encoded an HIV antigen and plasmid that encoded a mutated form of DR5.

7. Data in the manuscript show mice receiving the combination of plasmid that encoded Antigen and plasmid that encoded non-mutated DR5 exhibited enhanced immune responses compared to those observed in mice that received plasmid that encoded Antigen, or those that received the combination of plasmid that encoded Antigen and plasmid that encoded mutated DR5.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 1/20/06

By: _____
Dr. David B. Weiner

Exhibit 1

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DR5 triggered apoptosis functions as a vaccine adjuvant by inducing caspase-8 dependent dendritic cell maturation.

[1] Michael A. Chattergoon, [2] Yutaka Tamura, [1] Mathura Ramanathan, [1] Jason P. Shames, [1] Vera Saulino, [3] Norihiko Matsumoto, [1] Tara M. Robinson, [1] Karuppiah Muthumani, [4] Luis J. Montaner, & [1] David B. Weiner.

[1] Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia PA 19104 USA
[2] National Institute of Advanced Industrial Science and Technology, Toyohira-ku, Sapporo 062-8517, JAPAN.
[3] Chiba Institute of Technology; Narashino, Chiba 275-0016, JAPAN.
[4] The Wistar Institute, Philadelphia PA 19104, USA

Address correspondence to:
David B. Weiner
Associate Professor,
Dept. of Pathology and Laboratory Medicine,
University of Pennsylvania, School of Medicine.
422 Curie Blvd, 505 Stellar-Chance Laboratories
Philadelphia PA 19104.
Phone: 215-349-8591
FAX: 215-573-9436
E-mail: dbweiner@mail.med.upenn.edu

Key words: TNF receptor, apoptosis, vaccine, adjuvant.
Running Title: DR5 is an immune adjuvant.

Abstract.

Non-homeostatic tissue apoptosis *in vivo* has been shown to induce inflammatory responses and facilitate the cross-presentation of proteins within apoptotic bodies. We hypothesize that in the presence of foreign antigens, the apoptotic-inflammatory process improves immune priming; further, molecules that trigger apoptosis may be adapted for use as immune adjuvants. One very attractive molecule in this context is the TNFR family molecule DR5/TRAIL-R2. We show a significant improvement in $CD8^+$ T-cell mediated vaccine immunity with DR5 as an immune adjuvant, a property that is dependent on its ability to induce apoptosis in vivo which in vitro is correlated with activation of Caspase 8.

Introduction.

The manner in which apoptotic cells are treated by the adaptive immune system remains controversial. While apoptotic bodies (the remnants of cells undergoing programmed cell death) have been shown to transfer proteins/antigens (Ag) to phagocytes, including dendritic cells (DC), it remains controversial as to whether these proteins are exclusively utilized to maintain immune tolerance or whether they can also be used to initiate immune responses. There are reports to support both arguments, with more recent data demonstrating that proteins transferred to DC within apoptotic bodies appear to gain access to the cross-presentation pathway very efficiently; where their presentation has been associated with both the maintenance of tolerance as well as the induction of protective immunity (1-6). The central factor(s) determining whether Ag contained within apoptotic bodies will be immuno-stimulatory or -suppressive appears to be in the manner in which the apoptotic bodies were formed and delivered to DC. Apoptotic bodies formed by cell stressors, extrinsic signaling or in the presence of inflammatory molecules appear to be immune stimulatory (1-4, 7-19), whereas apoptotic bodies formed after DNA damage appear to be tolerizing (4, 20-22). This suggests that apoptotic bodies generated *in vivo* under particular conditions could be utilized to deliver Ag to DC in an immune-stimulatory form for the purpose of engineering potent CD8+ T-cell immune responses and useful immuno-therapies. Accordingly, the identification of molecules that could play a role in such a process is likely to be of interest.

Death Receptor–5 (DR5) (TNFR-SF10B) is a 411 amino-acid protein that activates the extrinsic pathway of apoptosis, thereby leading to the death and clearance of cells. The primary 4.4kb transcript of DR5 is found in many tissues of relevance to the immune system, including including the lung, gut and secondary lymphoid tissue (23, 24). However, DR5 protein expression has not been widely documented, limiting the study of this receptor. Notwithstanding this limitation, the study of this receptor and its role in the immune system remains of considerable interest, because the expression of DR5's only identified ligand, TNF-related Apoptosis Inducing Ligand (TRAIL), is upregulated by several tumors. Raising the possibility that tumors use TRAIL as a protective mechanism to avoid immune surveillance, in a manner similar to the mechanism employed by tissues found in "immune-privileged" sites. Such a system suggests that TRAIL is normally used in immune surveillance, implicating DR5 as a key molecule in this process. Further confounding the study of DR5 is the surprising observation that the systemic administration of TRAIL into mice does not cause significant pathology in animals (25). Like the Fas-FasL system, a homo-trimeric TRAIL complex interacts with a trimerized receptor initiate apoptosis (26); and like FasL the expression of TRAIL is very limited, only some NK subsets, activated CD8 T-cells (27, 28) and freshly isolated blood DC have been observed to express TRAIL (29). However, TRAIL expression can be induced on peripheral blood T-cells by IFN-α, β and γ (30), on NK cells by IFN, IL-2 and IL-15 (31), and monocytes and DC differentiated *ex vivo* by IFN (32, 33). This pattern of TRAIL expression suggests that DR5 may play a prominent role in the innate immune system, perhaps as an effector mechanism or as a bridge between the innate and adaptive immune systems.

We report here, that [1] DR5/TRAIL-R2 is a potent immune adjuvant in the DNA vaccine model. Further, [2] the adjuvant activity of DR5 is dependent on the pro-apoptotic death domain (DD) to induce apoptosis *in vivo*. [3] DR5 appears to be highly potent among TNFR-SF molecules in its ability to drive CD8+ T-cell responses. These studies suggest an interesting role for the TRAIL-DR5 system as a possible mechanism employed by the innate immune system for surveillance and activation of adaptive immune responses, particularly CD8+ CTL activity. We also demonstrate that apoptotic fragments generated after caspases-8 triggering can induce DC maturation a property not shared by apoptotic fragments generated after caspases-9 triggering.

Materials and Methods.

Plasmid DNA preparation.
The plasmid pcEnv expressing the HIV-1_{MN} gp160 (34), pHA expressing the Influenza A/PR8/34 HA (35) and the adjuvants murine pIL-12 (36), pTNFR-1 (37) and pFas (38) were previously described. The plasmids pCasp8 and pCasp9, expressing caspases 8 and –9 respectively were the kind gift of Dr J. Cohen (39). The TRAIL receptor, murine DR5 was cloned from a murine splenocyte total cDNA library by PCR using the forward primer 5'-GCCCCCAAGCTTGCCGCCACCATGGAGCCTCCAGGACCC-3' and the reverse 5'-ATCGGGCTCGAGTCAAACGCACTGAGATCCTCC-3'. The PCR product was digested to release a 1146 bp HindIII-Xho1 fragment. The truncated DR5 (tDR5) was generated by PCR using the previously described forward primer and the reverse primer 5'-ATCGGGCTCGAGTCATTAGAGCAACCATTGCCTCCATGCTCC-3' which introduces a premature stop codon into the mRNA transcript. The PCR product was digested to release a 630 bp HindIII-Xho1 fragment. These fragments were inserted into the pVax vector (Clontech, Palo Alto CA). Plasmids were purified using Qiagen Endo-Free Maxi Prep® kits (Qiagen, Santa Clara CA). Ox40 was cloned by a similar strategy using the forward primers 5'-GCCCCCAAGCTTGCCGCCACCATGGAAGGGGAAGGGGTTC-3' and the reverse 5'-ATCGGGCTCGAGTCACAGTGGTACTTGGTTCACAG-3'. Similarly RANK was cloned by a similar strategy using the forward primers 5'-GCCCCCAAGCTTGCCGCCACCATGGCCCCGCGCG-3' and the reverse 5'-ATCGGGCTCGAGTCATTCTGCACATTGTCCGG-3'.

Animals.
Female BALB/c mice 6-8 weeks old (Harlan Sprague Dawley, Inc., Indianapolis IN) were used throughout the experiment. All animals were housed in a temperature-controlled, light-cycled facility at the University of Pennsylvania, and their care was under the guidelines of the National Institute of Health and the University of Pennsylvania.

Immunization with plasmid DNA.
For immunology studies a standard protocol was used to prime the animals. Groups of four mice were immunized twice with pcEnv (100 μg) alone or pcEnv (100 μg) + Adjuvant (100 μg) on days-0 and 14. All immunizations were delivered into the quadriceps muscles in a total volume of 100 μ□ PBS (Gibco/LTI, Grand Island NY). On day-28 all animals, except those in the control group (vehicle control), were boosted with 100 μg pcEnv alone. The animals were sacrificed on day-35, 7 days after the last immunization, whereupon serum and the spleen were collected for immunology assays. For TUNEL analysis of the muscle, mice were immunized once with pcEnv (100 μg) or pcEnv (100 μg) + Adjuvant (100 μg) on day-0. On day-5 animals were sacrificed and the quadriceps muscle previously immunized were harvested and cryo-preserved. For challenge studies, groups of ten mice were immunized twice with pHA (33 μg) alone or pHA (33 μg) + Adjuvant (100 μg) on day-0 and day-14. On day-28 all animals, except those in the control group (vehicle control), were boosted with 33 μg pHA alone. The animals were rested for 150 days after the last immunization whereupon they were challenged with live Influenza A/PR8/34.

ELISpot Assay.
Splenocytes from immunized mice were stimulated *in vitro* and the number of responder cells secreting IFN-γ measured by ELISpot assay. The ELISpot plates are prepared 24 hrs in advance of the splenocyte harvest by coating 96-well nitrocellulose membrane plates (Millipore, Bedford MA) with an IFN-γ capture antibody (R&D Systems, Minneapolis MN); 4°C, overnight incubation per the manufacturer's recommendation. Following antibody coating, the plates are washed 6X with 200μl/well of PBS. Two hundred thousand splenocytes were added to each well along with 100 μl of rgp160 (2 μg/ml) (Protein Science Corp, Meridian CT), a pool of 212 peptides spanning the gp160 protein (15-mer with 11 a.a.

overlap), a pool of 122 peptides spanning the p55 protein (NIH AIDS Research and Reference Reagent Program, Rockville MD), Concanavalin A (10 μg/ml) (Sigma, St Louis MO) or R5 media (RPMI 1640 (Gibco/LTI) supplemented with 5% FCS (Hyclone, Logan UT), 2 mM L-Glutamine, 50 μg/ml Penicillin and 50 μg/ml Streptomycin (Gibco/LTI). Each condition was tested in triplicate. The plates were incubated at 37°C for 24 hrs to allow IFN-γ secretion and capture on the membranes. The plates were next washed as described and 100μl of the biotinylated anti-IFN-γ detection antibody (R&D Systems) added to each well at a concentration of 1.0 μg/ml in PBS, 4°C for 24 hrs. The plates were washed as previously described and 100μl of Streptavidin-AP (1.0 μg/ml in PBS) (R&D Systems) added to each well; room temperature for 2 hrs. Finally the plates were washed 6X with 200μl/well of PBS and 100 μl of the AP substrate solution BCIP/NBT (R&D Systems) added to each well; 30 min room temperature. The developing solution is removed by washing the plate with water.

Cytotoxic T lymphocyte Assay.
A six-hour ^{51}Cr release assay was performed using vaccinia-infected targets to measure lytic potential among the splenocytes (40). Splenocytes (effectors) from immunized mice were expanded *in vitro* for seven days, by first culturing in the presence of Concanavalin A (Sigma) (5μg/ml) for 48 hrs then with stimulator cells for the remaining five days. Stimulators were prepared by on day 3 by infecting P815 cells (ATCC, Manassas VA) at a multiplicity of infection of 10-20 for 12h at 37°C with vMN462 *env*-recombinant vaccinia (NIH AIDS Research and Reference Reagent Program). After infection the stimulators were fixed in 0.1% Glutaraldehyde (FisherBiotech, Fair Lawn NJ) and blocked in 0.1M Glycine (FisherBiotech). Vaccinia infected targets (vV1174 *env*-recombinant or vWR wildtype) (NIH AIDS Research and Reference Reagent Program) were prepared by infecting 3 x 10^6 P815 cells at a multiplicity of infection of 10-20 for 12h at 37°C. Immediately before addition to the effector cells the target cells were pulsed for 2 hrs with $Na_2{}^{51}CrO_4$ at 10 μCi/ml then washed extensively. CTL lysis was determined at E/T ratio ranging from 100:1 to 12.5:1 in a 96-well round-bottomed plate (BD Biosciences, Franklin Lakes NJ) (6 replicates per condition). Following an 6 hr incubation 50 μl of supernatant was harvested and added to 150 μl of OptiPhase Supermix scintillation fluid (Perkins Elmer, Downers Grove IL). The ^{51}Cr released into the supernatant was counted using a 1450 Microbeta Liquid Scintillation Counter (Perkins Elmer). The *Percent Specific Lysis* was calculated using the following relationship: % Specific lysis = 100 x (CPM specific lysis – CPM spontaneous lysis)/ (CPM maximum lysis – CPM spontaneous lysis).

CD8+ T-cell depletion.
In some cases CD8+ cells were depleted from the effector population prior to the ELISpot Assay and CTL assay. CD8+ cells were positively selected and removed using the CELLection Mouse CD8 Kit # 114.09 (Dynal Biotech, Lake Success NY) per the manufacturer's recommendations.
In vaccine challenge experiments, animals were depleted of CD8+ T-cells *in vivo* prior to infection. This was achieved by i.p. injection with the monoclonal antibody 53-6.7 (0.5 mg/mouse). This removes 98% of all circulating CD8+ T-cells and 92% of all splenic CD8+ T-cells within 48 hours (data not shown).

Lymphoproliferation Assay.
For analysis of recall proliferation to antigen, $5x10^5$ splenocytes were cultured at 37°C in a 96-well round-bottomed plate (BD Biosciences) with rgp160 (2 μg/ml), rp55 (2 μg/ml) (Protein Sciences Corp), Concanavalin A (5 μg/ml) or R5. Six replicates of each sample were conducted. Following 72 hrs incubation, each sample was pulsed for 12 hrs with 1 μCi ^3H-Thymidine (NEN, Boston MA), harvested and CPM determined using a 1450 Microbeta Liquid Scintillation Counter (Perkins Elmer). The stimulation index (SI) is defined as the ratio of the CPM upon stimulation with rgp160 or Concanavalin A versus R5 alone.

Flow cytometry determination of caspase activity.
To determine of the apoptotic potential of pDR5 and ptDR5, RD cells (ATCC) were seeded at $5x10^5$ cell/plate on 60 x 15 mm tissue culture treated plates (BD Biosciences) and transfected with the pVax plasmid or plasmids expressing DR5 or the mutant tDR5. After 36 hrs incubation the cells were gently re-suspended with VERSENE, washed twice in 1X PBS and the *in situ* marker CaspACE FITC-VAD-FMK (Promega, Madison WI) added per the manufacturer's protocol. After 15 min incubation the cells were washed extensively with 1X PBS and analyzed directly on a Coulter EPICS® Flow Cytometer (Coulter, Hialeah FL). Immediately prior to analysis Propidium Iodide (0.5 μg/ml) (Sigma) was added to each sample.

Colometric determination of caspase activity.
To simultaneously measure active caspase-3, -8, and –9 in cells, RD cells were transfected as described previously with pDR5, pCasp8, pCasp9 and pLacZ in a 4:1 ratio. After 18 hrs, the cells were harvested, lysed and developed with a colometric substrate for active caspase 3 (Caspase –3 Colometric Assay Kit, MBL, Woburn MA), active caspase 8 (Caspase–8 Colometric Assay Kit, MBL, Woburn MA), and active caspase 9 (Caspase–9 Colometric Assay Kit, MBL, Woburn MA), per the manufacturers's protocol. As a control, some cells were incubated with Actinomycin-D (30μg/ml) for 12 hrs. Prior to measurement of caspases activity the samples were adjusted for transfection efficiency by adjusting to OD 405nm of 0.5

TUNEL staining.
Cryo-preserved muscle sections were probed for the presence of DNA breaks, a characteristic of apoptotic cells, using the TUNEL technology. The tissue sections were developed using the *in situ cell death detection kit* (Roche Applied Science, Mannheim Germany) per the manufacturer's recommendations. Briefly, the slides were fixed in freshly prepared 4% Paraformaldehyde (Sigma) then permeabilised with 0.1% Triton X-100, 0.1% Sodium Citrate. The slides were washed extensively, and incubated for 60 min with the TUNEL reagent. DAPI (Sigma) was then added for 5 min, after which the slides were washed and cover glasses placed.

Infection with Influenza A.
The animals were temporally anesthetized with Ketamine/Xlyazine (conc) given by i.p injection and subsequently handled for Influenza A/PR8/34 infection. The animals are given 1.0 HAU of live Influenza A/PR8/34 in 50 μl PBS by direct intranasal administration via. pipette droplets placed on the nostrils.

Results.

DR5 significantly enhances priming of CD8$^+$ antigen-specific CTL *in vivo*.

We studied the ability of three DD containing TNFR-SF members (Fas, TNFR-1 and DR5) to enhance the priming and expansion of MHC class-I restricted CTL responses *in vivo*. CTL activity was measured in mice immunized with pcEnv (expressing HIV-1_{MN} gp160) alone, or in combination with pDR5, pFas and pTNFR-1. As a control, some animals were immunized with pcEnv and the adjuvant vehicle plasmid pVax (Figure 1a). HIV-1 gp160 specific CTL were measured as described in Methods.

In agreement with previous observations (38), immunization with pcEnv primes CD8$^+$ CTL that specifically kill target cells infected with recombinant vaccinia expressing HIV gp160 (solid symbols, VP1174) when compared to cells infected with wildtype vaccinia (open symbols, vWR) at E/T ratios of 100:1, 50:1. Co-immunization with pcEnv + pFas increased Ag-specific CTL lysis 2-3 fold over mice primed with pcEnv alone, and 5-fold over naïve levels at the E/T ratios of 50:1 and 25:1 (Figure 1b). TNFR-1 increased Ag-specific CTL approximately 2-fold over pcEnv immunized mice (Figure 1d). However, the best result was obtained with the pDR5; in this case a 3-5 fold increase in Ag-specific CTL response at the E/T ratios of 100:1, 50:1 and 25:1 was noted as compared with pcEnv immunized mice and approximately 7-fold over naïve mice (Figure 1c). These data indicate that DR5 is the most potent immune adjuvant among DD containing TNFR-SF members.

We also compared this activity with non-apoptotic, non-DD containing TNFR-SF molecules. Both pRANK (Figure 1e) and pOx40 (Figure 1f) failed to enhance the priming of CTL when used in conjunction with pcEnv. In each case the CTL activity was indistinguishable from that of mice immunized with pcEnv alone, suggesting an inability of these molecules to improve CTL priming *in vivo*. To confirm the adjuvant effect on CD8$^+$ T-cell priming, CD8$^+$ cells were depleted from the effector pool immediately prior to the lytic assay. Depletion of CD8$^+$ cells effectively abolished Ag-specific lysis of labeled targets (*data not shown*). These studies demonstrate that DR5 has an unusual potency among TNFR-SF members in directing the expansion of a CD8$^+$ CTL response.

DR5 significantly enhances priming of antigen-specific IFN-γ CD8$^+$ T cell *in vivo*.

We next investigated the ability of DR5 to modulate the frequency of IFN-γ producing CD8$^+$ T cells primed by vaccination. As in previous experiments, mice were immunized with pcEnv alone or in combination with pFas, pDR5, pTNFR-1, pRANK and pOx40. One week after the last immunization, splenocytes were harvested and re-stimulated *in vitro* with rgp160 (data not shown) or a peptide pool of 15-mers spanning the gp160 protein (Figure 2) in an ELISpot assay. The number of IFN-γ□□□□□□□ng T cells was quantified as described in Methods. Among splenocytes stimulated with the gp160 peptide pool, three immunizations with pcEnv induced 271 ± 85 SFU/10^6 cells (Figure 2a). The TNFR-SF adjuvants pTNFR-1 and pFas adjuvant increased this frequency 1.5-fold (420 ± 135 SFU/10^6 cells) and 2.3-fold (636 ± 72 SFU/10^6 cells) respectively over mice immunized with pcEnv alone ($p<0.005$). However, when co-immunized with pDR5, gp160-specific IFN-γ T cells increased to 1571 ± 85 SFU/10^6 cells, which represents a 5.7-fold increase over the number of T cells primed after multiple immunizations with pcEnv. As in the killing assay, no improvement in vaccine response was observed when splenocytes from pcEnv + pRANK or pcEnv + pOx40 groups were re-stimulated with rgp160 or the gp160-peptide pool (Figure 2b).

ptDR5 is unable to induce apoptosis.

The data above suggests that the DD is likely playing a role in enhancing CD8 T cell priming and subsequent effector activity. In order to determine whether apoptosis is central to the adjuvant properties of pDR5 we constructed a mutant DR5 in which the cytoplasmic tail is truncated prematurely, thereby removing the DD of the molecule and removing its ability to interact with the pro-caspase molecule. The

630 bp truncated DR5 (tDR5) was fully sequenced and cloned as described in *Methods*. The expression of both tDR5 and DR5 constructs were determined by *in vitro* translation (Figure 3a). As shown, pDR5 encodes a 30kD protein, while ptDR5 encodes an 18kD mutant whose function was confirmed as follows.

To eliminate disparate protein expression levels as a trivial explanation for a loss/gain of function in the truncation mutant, we examined DR5 expression in transfected cells. In this experiment, RD cells were transfected with pDR5 and ptDR5 then cultured both in the presence of a caspase 3 inhibitor (abolishing any apoptotic signals from the transfected plasmids). As shown in Figure 3b, in the presence of the caspase-3 inhibitor both wildtype and tDR5 are expressed at similar levels. In both cases approximately 15-17% transfection efficiency is observed with MFI = 11.3 for DR5 and MFI = 9.4 for tDR5.

We confirmed the biological activity of pDR5 and ptDR5 by transfecting RD cells and directly measuring caspase activity and cell viability. Expression of the pDR5 led to apoptosis of transfected cells with differences in expansion rate of the pDR5 and control cultures observed as early as 18 hrs (*data not shown*). At 36 hrs, 29.6% of the pVax transfected cells captured the FITC-labeled caspase substrate VAD-FMK (Figure 4a), while 70.4% fail to bind the caspase substrate indicating a lack of active caspase in these cells. In cultures transfected with pDR5 the live/dead is reversed at 36 hrs: 60.8% being VAD-FMK$^+$ and 39.2% VAD-FMK$^-$ (Figure 4e). ptDR5 transfected cultures were similar to the pVax transfected cultures indicating that the pro-apoptotic ability of DR5 is lost by the truncation (Figure 4i). This effect was more noticeable upon the addition of propidium iodide to cultures at 36 hrs; 20.7%, 49.3% and 20.6% of cells in pVax, pDR5 and ptDR5 respectively were permissive to propidium iodide indicating that they have disrupted plasma membranes (Figure 4b, f, j).

The loss of function mutant ptDR5 was also confirmed *in vivo*. Muscle sections isolated from the prior immunization site on the fifth day post-immunization were frozen and cryo-sectioned as described. The tissue was then probed with DAPI and developed by TUNEL. Figure 4 demonstrates the ability of pDR5 to induce apoptosis at the site of immunization. In mice immunized with pcEnv alone no significant TUNEL staining was observed (Figure 4c, d), suggesting the immunogen plasmid and the process of immunization does not lead to significant tissue death. The addition of pDR5 led to significant cell death at the immunization site, corresponding to large areas of TUNEL positive cells (Figure 4g, h). This was further confirmed by immunization with pFas, which also induces large areas of TUNEL positive cells at the immunizations site (*data not shown*). Further, as predicted, muscle immunized with the DD truncated ptDR5 were not TUNEL stained (Figure 4k, l). Taken together these data suggest that the DD truncation generates a non-signaling, non-apoptotic, dominant negative mutant.

Adjuvant properties of pDR5 segregate with the DD.

To directly determine whether the adjuvant properties of pDR5 are related to its ability to induce apoptosis we immunized animals with the ptDR5 as an adjuvant. This was compared directly with pDR5 as a positive control in this assay. Splenocytes from immunized mice were tested for gp160-specific lytic activity as described previously. Similar to prior assays, addition of pDR5 at the priming steps generates strong lytic activity to vaccinia-gp160 infected targets, greater than 60% at E/T = 100:1 (Figure 5a). In mice primed with ptDR5 the gp160-specific lytic activity was similar to animals immunized with pcEnv alone less than 25% at E/T = 100:1. This differential effect on immune priming was further explored by counting the gp160-specific responder frequency after priming. Again pDR5 increased antigen specific IFNγ ELISpot (1381 ± 172 SFU/10^6 splenocytes) upon re-stimulation of splenocytes harvested from animals immunized with pcEnv + pDR5. Truncation of the DD led to a loss of DR5 adjuvant properties: IFNγ ELISpot response falls to the pcEnv alone levels: 475 ± 197 SFU/10^6 splenocytes vs. 420 ± 135 SFU/10^6 splenocytes ($p<0.05$) (Figure 5b).

We next looked at whether expression of the pDR5 and the ptDR5 molecule, lacking the DD, could drive expansion of CD4$^+$ T cell LPA responses in a manner similar to CTL expansion. Here we find that recall

proliferation to the vaccine antigen was greatly enhanced when pDR5 was used as a DNA adjuvant (SI of 14.65 ± 2.61 vs. pcEnv + pDR5). Recall proliferation to the vaccine antigen was greatly reduced with pcEnv + ptDR5 (SI = 3.91 ± 0.99) (Figure 5c). This suggests that the DR5 adjuvant activity for both $CD4^+$ and $CD8^+$ T cell expansion is largely inter-dependent on the DD function of this molecule.

pDR5 adjuvant generates long lasting immunity.

To assess the longevity of the immune response after priming with pDR5 animals were immunized as described previously with pcEnv and pcEnv + pDR5 or pcEnv + ptDR5. At days 7, 30, 60, 90, 120, 150, 180 and 360 (data not shown for days 60, 120, 180 and 360) after the last immunization, splenocytes from the immunized mice were tested for lytic activity and CTL precursor frequency. The general pattern of lytic activity in response to challenge with gp160-vaccinia infected cells was the same in each cohort: a peak response was recorded on day-7 post-immunization, after which the magnitude of the lytic activity quickly fell to a steady state level. Once established, the steady state level was maintained up to 360 days after the last immunization (Figure 6a). On each observation day, the lytic activity in animals primed with pcEnv + pDR5 was at least 2.5-fold greater than the level maintained in animals primed with pcEnv alone.

This pattern was closely followed when the frequency of $CD8^+$ T cell responders was counted in the period following the immunizations. Seven days after the last immunization, gp160-specific IFN-γ ELISpot was >3-fold increased (1284 ± 238 SFU/10^6 splenocytes) in mice given the pDR5 adjuvant as compared to mice co-immunized with ptDR5 (410 ± 113 SFU/10^6 splenocytes) or with no adjuvant (379 ± 130 SFU/10^5 splenocytes). Interestingly, although the overall number of responders falls to a steady state level as early as day-30 post-immunization, the overall ratio of the expansion is preserved. For example at day-150 post immunization responder frequency in the pcEnv + pDR5 (466 ± 81 SFU/10^6 splenocytes) is 3.13 greater than animals receiving pcEnv alone (149 ± 27 SFU/10^6 splenocytes). On each day post-immunization the ratio of the response between animals primed with pDR5 and ptDR5 was preserved (Figure 6b). Theses data demonstrate that the use of DR5 during vaccine priming can have a significant impact on immune memory and cellular vaccine efficacy.

pDR5 adjuvant confers vaccine protection.

The longevity of the immune response primed by DR5, animals was confirmed in animals primed with pHA. As described previously mice were immunized with pHA and pHA + pDR5 or pHA + ptDR5. At days 7, 90 & 150 after the last immunization the CD8+ T-cell responder frequency was tested by challenging splenocytes with live Influenza virus. As observed with pcEnv on each day post immunization the ratio of HA specific CD8+ T-cells between animals primed with pDR5 and ptDR5 was consistently >3-fold as was the ratio of the response between animals primed with pDR5 verses no adjuvant at all (Figure 7a).

The ability of the pDR5 to confer vaccine protection in a virus challenge model was tested in an influenza challenge model. Groups of 20 animals were vaccinated i.m. with the pHA plasmid (encoding the Influenza A PR8/34 Hemagluttinin molecule) alone, or in combination with pDR5, ptDR5 or pVax as described in *Materials and Methods*. The animals were then rested until 147 days post-immunization when one cohort of 10 animals was depleted of CD8+ T-cells, both depleted and non-depleted cohorts were challenged 3 days later with 1.0 HAU live Influenza A PR8/34 (day 150).

The non-depleted, naïve animals become infected rapidly, showing rapid weight loss and labored breathing within the first three days post-infection. These animals were followed for 12 days at which time the animals had lost approximately one-half of their starting body weight (Figure 7b). Animals immunized with the pHA plasmid alone show similar weight loss characteristics initially, but then move along a more protracted weight loss curve eventually reaching a nadir approx –7.06 ± 1.89 gm representing a 25-30% loss in total body weight. Animals receiving the pHA + ptDR5 followed a very

similar weight loss curve as those animal receiving the antigenic plasmid alone. Although these animals only reach a nadir of –5.38 ± 1.56 gm (approx 20% loss in total body weight), the behavior of the cohorts is not statistically different from the pHA alone cohorts at any time-point post-immunization. Remarkably, the cohort receiving pHA + pDR5 showed complete control the infection. In addition to showing no significant weight loss as compared to the pre-challenge weight, there were also no visible signs of infection. Antibody levels did not change and increases in CD8+ T-cells were observed.

As we have previously demonstrated a strong effect of pDR5 on T-cell priming, we tested challenge outcome if CD8+ T-cells were depleted in vivo prior to challenge. CD8+ T-cell depletion was performed and confirmed 3 days after i.p. injection of 0.5 mg of the mAb 53.1-6 (data not shown), at which time the animals were challenged as described previously. In contrast to the non-depleted cohort, the depletion of CD8+ cells completely abolished any protection conferred by vaccination (Figure 7c). These cohorts behaved like the naïve animals, rapidly establishing infection and exhibiting signs of morbidity. These studies support a long-term memory benefit to CD8+ T-cell responses generated by DR5.

Apoptotic bodies generated through caspase-8 activation have adjuvant properties.
Finally, we sought to confirm our prediction that DR5 activates caspase-8 selectively, and that the adjuvant properties we have observed thus far are related to caspase-8 activation. We used a spectrophotometric method which allows simultaneous detection of the relative concentrations of active caspase-8, -9 and -3 to study the activity of DR5 on the effector-caspases. In this experiment, RD cells were transfected with pLacZ alone or in combination with pDR5 or pCasp8 and pCasp9 using standard transfection techniques. The cells were then allowed to incubate for 18-hours, they were then lysed to release their cytoplasmic contents. Using the colormetric LacZ substrate the concentration of each sample was adjusted to give a LacZ activity of OD_{405nm} 0.5, in this manner the transfection efficiency of each sample was standardized prior to the measurement of active caspases.

The control plasmids pCasp8 and pCasp9 induced caspase-8 or caspase-9 respectively, the activities of which were easily detected and in each case was associated with the activation of caspase-3 (Figure 8A). Actinomycin-D also induced active caspase-9 and caspase-3 in this system as previously reported (ref). These data indicate that both caspase-8 and –9 are active in RD cells and are capable of inducing caspase-3. Notably DR5 expression by the pDR5 plasmid led an almost selective activation of caspase-8 when compared to caspase-9, with a relative ratio of 18:1, further this was associated with the activation of caspase-3 and apoptosis.

We then collected the supernatant fraction from similarly transfected cultures, and added them to cultures of *in vitro* derived bone marrow dendritic cells, prepared previously following established protocols (41). After 24-hrs the dendritic cells were analyzed by flow cytometry for the surface expression of CD86 and MHC-II on CD11c+ cells as an indication of DC maturation. As shown in Figure 8B, apoptotic fragments generated by transfection with pCasp8 induce an increase in MHC-II and CD86 in a subset of the population of CD11c+ cells. Similar changes in MHC-II are changes associated with maturation of dendritic cells. Fragments generated with pCasp9 do not activate dendritic cells in the same manner despite similar caspase-3 activity. These data suggest that apoptosis triggered by caspase 8 has a different consequences for the immune system as compared to caspase 9. Predictably pDR5 which selectively activates caspase-8, appears to be associated with dendritic cell maturation.

Discussion

DR5 is a particularly interesting member of the TNFR-SF as its ligand TRAIL is an important death-inducing molecule, which is primarily expressed by cells of the innate immune system, including NK and DC(29, 31-33, 42). TRAIL mediated cytotoxicity is thought to be very important in the immune surveillance of tumor cells (43); however, the importance of TRAIL-DR5 in clearance of infected cells is not well defined. Furthermore, there is no current understanding of a link between DR5 apoptosis and

adaptive immunity. Although limited, the expression of TRAIL is well suited/designed to facilitate the delivery of intra-cellular antigens to DC and the adaptive immune system. Such a system would rely on infected cells up-regulating their expression of DR5, in this way DC could directly kill infected cells then acquire and present their antigens. Alternatively, the pre-formed and fast-acting effectors of the innate immune system, such as NK cells, kill infected cells and generate apoptotic fragments that are taken up and presented by DC. This also implies that the up-regulation of DR5 is carefully controlled and potentially linked to cell stress or other viral sensors. Finally, this model hints that apoptosis initiated by via. caspase 8 activation (which requires and ligation of the DR by a second party) may have different immune consequences as compared to apoptosis initiated by caspase 9 (requiring no external stimuli), perhaps leading to the release of immune activating factors which fully capacitate DC (Figure 8). Such a system would allow the immune system to distinguish between homeostatic and non-homeostatic apoptosis. Intriguingly, apoptotic bodies formed by caspase 3 activation have been shown to have chemo-attractant properties (44). However, further clarifying experiments are needed explore this possibility.

Our data showing improved T-cell responses to vaccine antigens upon priming with pDR5 suggests a link between DR5 and the adaptive immune system. We hypothesize that the adjuvant mechanism of DR5 could involve two separate and complementary mechanisms. When plasmids alone are injected, myocytes at the site of immunization are transfected and become the major source of antigen. However, this protein is largely sequestered by the myocyte and is only released upon the arrival of primed effectors at this muscle, these cells view the myocyte as "infected" and lyse the cell. Thus, in the absence of adjuvants like DR5 most if not all antigen presentation and immune priming probably occurs by direct transfection of DC with minor contributions from cross-priming pathways (45, 46). On the other hand if DR5 is expressed, the transfected muscle cell is inclined to undergo apoptosis (Figure 4) by mechanisms that involve the over-expression of DR5 and ligand triggering. This generates apoptotic fragments containing the antigen which can later be internalized by DC that were not among the cohort directly transfected. Thus with DR5 the contribution to immune priming by the cross-presentation pathway may be a significant and additive. We tested this hypothesis by constructing a dominant negative form of DR5. This molecule has a truncated cytoplasmic tail and is unable to initiate apoptosis (Figure 4). We expect that if apoptosis were central to the adjuvant activity of DR5, the mutant would be functionally inactive and would induce the same response as the pcEnv group alone. Indeed this was the case when ptDR5 was compared to the pDR5. Ag-specific $CD8^+$ T-cell priming was indistinguishable in animals given the plasmid immunogen alone and those that received the immunogen and truncated DR5 (Figure 5, Figure 6, Figure 7).

Extending this work to members of the TNFR-SF, we have found that several TNFR-SF molecules containing the DD are also adjuvants for DNA vaccines, however none are as potent as DR5. The molecules TNFR-1, Fas, DR5 and NGNF (*data not shown*) induced higher Ag-specific CTL (Figure 1) when used in combination with the plasmid expressing *env*. In particular, DR5 induces robust CTL activity five times greater than the level induced with the pcEnv plasmid alone and seven times above the level in unprimed mice. Further, this effect was consistent in two other Ag systems tested, HIV-1 *gag* (*data not shown*) and Influenza A/PR8 HA (Figure 7*a*). Again, the adjuvant effect was observed if IFN-γ secretion was measured upon antigen re-stimulation, DR5 priming led to significantly higher number of IFN-γ secreting responders (Figure 2). Interestingly the non-apoptotic, TRAF interacting molecules Ox40 and RANK did not boost CTL or responder frequency. Additionally, we have also studied CD40 and 4-1BB, which also failed to show any adjuvant properties for CTL (*data not shown*). It appears that the adjuvant property of TNFR-SF segregate with the presence of the DD motif. This result is particularly interesting as it confirms that non-homeostatic apoptosis of Ag bearing cells *in vivo* serves as a mechanism for the activation of DC and $CD8^+$ T-cell responses. Further, it suggests that regardless of the initial apoptotic trigger, receptor mediated, cell stress, etc. this mechanism may be an important adjuvant mechanism for intra-cellular pathogens.

The adjuvant mechanism of DR5 may be highlighting the importance of the cross-priming mechanism in ensuring DC exposure to microbes with limited tropisms. Immature DC are known to efficiently take up antigens in peripheral tissue and secondary lymphoid organs where they can present these antigens to T-cell after receiving maturation signals. DNA vaccines mimic intra-cellular organisms in the manner by which they induce immunity. However, since they are non-replicating, they display a "pseudo" tropism in that, only those cells which become transfected during the immunization, contain the plasmid and can produce the antigen for immune priming. This leads to a limitation in that only DC that were directly transfected during immunization can participate in vaccine priming. It is for this reason that we believe that tissue damage and the spread of antigen in apoptotic bodies allows further immune priming in DC that were not transfected, rather they use the cross-priming pathway. Thus, the induction of apoptosis and tissue injury may be intimately linked to the recognition of a foreign antigen. While this requires further investigation, we hypothesize that specific non-homeostatic apoptosis of tissues induces a conserved signaling mechanism normally initiated in response to tissue injury. By co-delivering an antigen and the DR5 pro-apoptotic molecule as an adjuvant, we generate apoptotic bodies containing foreign antigens which can be presented by DC, we hypothesize that a DC maturation signal is likely intrinsic to the DR5 death pathway. The generation of maturation factors (47-50) in this manner may be important, for the cross-priming pathways of DC (51-54). Such a mechanism may be especially relevant for the presentation of pathogens/antigens which are already causing tissue damage.

The role of DR5 in the immune system is not well understood and awaits the development of targeted knockout animals. We demonstrate here an unusual biology for this relatively unexamined member of the TNFR-SF. This molecule appears uniquely potent at driving immune expansion of CD8+ T-cells. Such a system may be very important in the immune response to viral infections or perhaps tumor clearance. Understanding the regulation of DR5 will have implications for understanding the transition from the innate to the adaptive immune response. The data supports a model where DR5 functions as a bridge between the innate and adaptive immune systems, and suggests that TRAIL is more than an immune system effector, perhaps it is an essential link between the innate and adaptive immune response. Already several reports have demonstrated the importance of TRAIL-DR5 in clearance of virus-infected cells (55, 56). Understanding how to harness this system may present important opportunities for therapeutic intervention in human inflammatory diseases.

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Figure Legends.

Figure 1. Pro-apoptotic TNFR-SF adjuvants prime strong CTL in immunized mice.

Groups of four mice were immunized with pcEnv alone or in combination with (a) pVax, the pro-apoptotic TNFR-SF adjuvants (b) pFas, (c) pDR5 and (d) pTNFR-1, as well as the non-apoptotic TNFR-SF adjuvants (e) pRANK and (f) pOx40 as described in *Methods*. Seven days after the last boost, animals were sacrificed and gp160-specific $CD8^+$ T-cells expanded *in vitro* for 7 days with Con A and fixed vaccinia-gp160 infected P815 cells. Specific CTL activity was determined by comparing the ability of re-stimulated effectors to kill *env(gp160)*-recombinant vaccinia (vP114) infected P815 cells (ν & λ) versus wild-type vaccinia (vWR) infected P815 cells (open, □ & μ) at varying E/T ratios in a 6 hr ^{51}Cr release assay. These data represent the average percent specific lysis and standard deviations of six independent experiments with similar outcomes.

Figure 2. pDR5 adjuvant increases Ag-specific responder frequency.

Groups of four mice were immunized with pcEnv alone or in combination with (a) the pro-apoptotic TNFR-SF adjuvants pTNFR-1, pFas and pDR5 as well as (b) the non-apoptotic TNFR-SF adjuvants pOx40 and pRANK as described in *Methods*. Seven day after the last booster, animals were sacrificed and the pooled splenocytes re-stimulated *in vitro* for 24 hrs with peptide pools spanning the gp160 protein (black) or p55 (grey) proteins of HIV-1, after which the number of IFN-γ secreting T-cells was determined as described in ELISpot methods. Stimulation with media alone (white) and Con A (*not shown*, SFU/10^6 splenocytes >2500) were used as controls. These data represent the SFU/10^6 splenocytes and standard deviations of six independent experiments with similar outcomes.

Figure 3. Deletion of the DR5 cytoplasmic tail.

(a) The cytoplasmic tail of DR5 was deleted at a.a. [redacted] to form a cytoplasmic tail truncation mutant, which was cloned into the pVax plasmid backbone. *In vitro* translation and immunoprecipitation with polyclonal anti-DR5 antibody was used to demonstrate the expression of this mutant. Lane A shows expression of the wildtype DR5 molecule that migrates at the predicted size of 30kD. Lane B; deletion of tDR5, deletion of the cytoplasmic tail generates a mutant that is approximately 18kD. (b) *In vivo* expression of the wildtype and truncation mutants was confirmed in RD cells. RD cells were transfected with pDR5 and ptDR5 and cultured in the presence of a caspases-3 inhibitor. Thirty-six hours after transfection, the cells were collected and incubated with polyclonal anti-DR5 antibody (white lines) and developed with a FITC-labeled secondary Ab (secondary Ab control shaded).

Figure 4. Deletion of the DR5 cytoplasmic tail leads to a loss of pro-apoptotic function.

The DR5 cytoplasmic tail deletion mutant was tested for pro-apoptotic function *in vitro and in vivo*. RD cells were transiently transfected with the vector backbone pVax (a, b), the wild-type pDR5 (e, f) and the mutant ptDR5 (i, j). Thirty-six hours after transfection the cells were incubated with the FITC-labeled caspase substrate VAD-FMK for 15 min (a, e, i), washed extensively and then analyzed by flow-cytometry. Immediately prior to analysis, Propidium iodide was added to further demonstrate dead cells in the culture (b, d, f). The gates in panels a, e and i, refer to the percentage of cells retaining a high and low level of the fluorescent caspase substrate. While the gates in panels b, f, and j refer to populations within the culture that are highly permissive or non-permissive to PI. The wildtype and truncated DR5 were also tested for their ability to induce apoptosis *in vivo*. Animals were immunized with pcEnv in combination with pVax (c, d), pDR5 (g, h) and ptDR5 (k, l) as previously described. Five-days after immunization the animals were sacrificed and the muscle at the site of immunization was harvested and cryo-sectioned. Apoptotic activity was measured by TUNEL staining *in situ* of the harvested tissue. Panels c, g, and k show tissue sections that were stained with DAPI to reveal the location of nuclei within the muscle fibers. Panels d, h, and l show consecutive tissue sections stained by TUNEL as described in methods.

Figure 5. DR5 DNA adjuvant activity segregates with apoptotic activity

To compare the pDR5 and ptDR5 adjuvants, mice were immunized with pcEnv, pcEnv + pDR5 or pcEnv + ptDR5 as described in *Methods*. Seven days after the last immunization splenocytes were harvested and compared for lytic activity, IFN-γ producing cells and recall proliferation. Antigen specific CTL activity was determined by comparing the ability of effectors from mice immunized with pcEnv + ptDR5 (v, □) or pcEnv + pDR5 (λ, μ) to kill *env*-recombinant (vP114) and wildtype (vWR) vaccinia infected P815 cells at varying E/T ratios in a 6 hr ^{51}Cr release assay. These data represent the average percent specific lysis and standard deviations of six independent experiments with similar outcomes. (a). The responder frequency of IFN-γ producing cells upon re-stimulation with peptide pools spanning the HIV-1 gp160 (black) and p55 (grey). Con A (not shown >2500 SFU/10^6 splenocytes) and media (white) were also determined in the splenocyte pool from vaccinated mice using the ELISpot assay as described in *Methods*. These data represent the average SFU/10^6 splenocytes as determined in six independent experiments (b). A 72-hr recall proliferation assay in response to rgp160 (black) and rp55 protein (grey) and media (white) stimulation was also performed on these samples. These data represent the average stimulation indices and standard deviations of six independent experiments with similar outcomes (c).

Figure 6. pDR5 generates long lasting immunity.

The ability of the pDR5 adjuvant to generate long lasting immunity was tested in the following manner. Groups of four mice were immunized with pcEnv (white) alone or in combination with pDR5 (black) or ptDR5 (grey) as described in *Methods*. (a) Seven, 30, 90 and 150 days after the last boost, animals were sacrificed and Ag-specific $CD8^+$ T-cells expanded *in vitro* for 7 days with Con A and fixed vaccinia-gp160 infected P815 cells. Specific CTL activity was determined by comparing the ability of re-stimulated effectors to kill *env*-recombinant vaccinia infected P815 cells versus wild-type vaccinia infected P815 cells, percent specific lysis was calculated as described in *Methods*. (b) The responder frequency of IFN-γ producing cells upon re-stimulation with peptide pools spanning the HIV-1 gp160 and p55 (not shown). Con A (not shown >2500 SFU/10^6 splenocytes) and media (not shown) were also determined using the ELISpot assay as described in *Methods*. These data represent the average SFU/10^6 splenocytes as determined in four independent experiments.

Figure 7. pDR5 confers protection after Influenza challenge.

The ability of the pDR5 adjuvant to generate long lasting immunity was confirmed using the Flu model. (a) Groups of four mice were immunized with pHA (white) alone or in combination with pDR5 (black) or ptDR5 (grey) as described in *Methods*. (a) Seven, 90 & 150 days after the last boost, animals were sacrificed and the responder frequency of IFN-γ producing cells upon re-stimulation with live Influenza (1 HAU/ml), Con A (not shown) and media (not shown) were also determined using the ELISpot assay as described in *Methods*. These data represent the average SFU/10^6 splenocytes as determined in four independent experiments. The ability of the pDR5 adjuvant to confer vaccine protection after priming was tested in an Influenza challenge model. Groups of 20 animals were divided into two cohorts each receiving by i.m. immunization the pHA plasmid alone (■), or in combination with pDR5 (λ) or ptDR5 (□); naïve animals were used as controls (□) as described in *Materials and Methods*. At 150 days post-immunization, (b) one cohort was challenged with 1.0 HAU live Influenza A PR8/34 by i.n. administration. The second cohort (c) was depleted of $CD8^+$ cells, and challenged 3 days later with 1.0 HAU live Influenza A PR8/34. The animals were monitored for signs of infection and weighted daily. The average change in total body weight for the cohort post-infection is shown.

Figure 8. Caspase 8 and DR5 generated apoptotic bodies which activate dendritic cells.

(a) The ability of the pDR5 to induce apoptosis through the activation of caspase-8 was confirmed using a spectrophotometric model. RD cells were transfected with p□□□□ alone or in combination with pVax, pDR5, pCasp8 or pCasp9 and left for 18 hrs. Additional p□□□□ transfected cells were treated with Actinomycin-D for 12 hrs. At this time cells were collected and lysed, the LacZ substrate was added to

each sample and the absorbance measured by spectrophotometer. Each sample was then adjusted to a LacZ activity of OD450nm 0.5. The developing reagents for caspase-3, -8 and –9 were added to standardized aliquots and the level of active caspases were measured using a spectrophotometer. (b) RD cells were transfected with pVax, pDR5, pCasp8 or pCasp9 as previously described; after 18 hrs the supernatant was collected and added to previously prepared bone marrow derived dendritic cells. After a 24 hr co-incubation, a cocktail of anti-CD11c-APC, anti-CD86-PE and anti-I-A^d-FITC was added to the cells for 30 min. The cells were analyzed by flow cytometry, by first gating on CD11c+ cells then analyzing the expression of MHC-II and CD86 on these cells.

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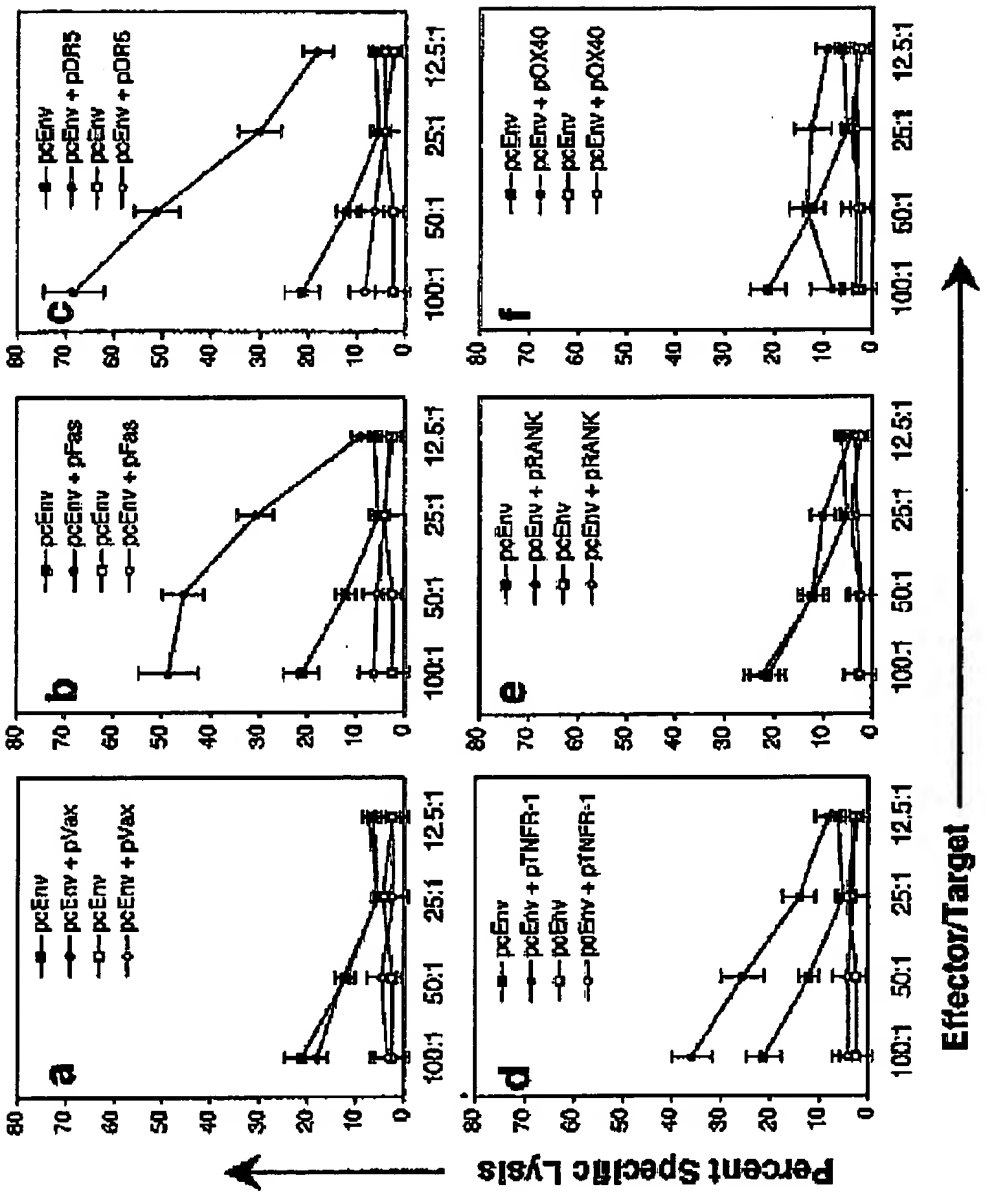
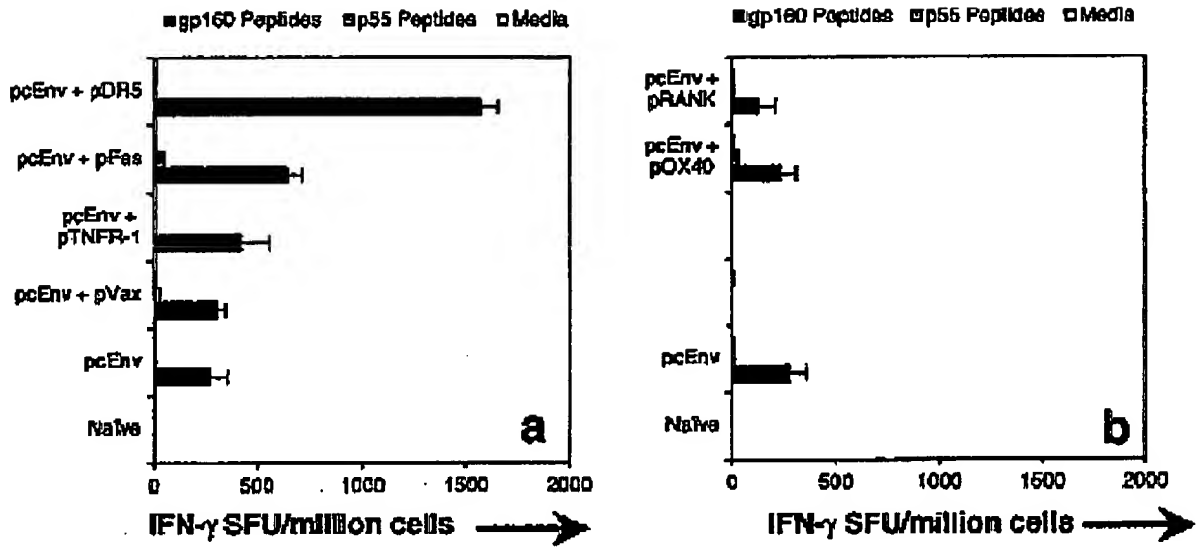


Chattergoon et. al. Figure 1



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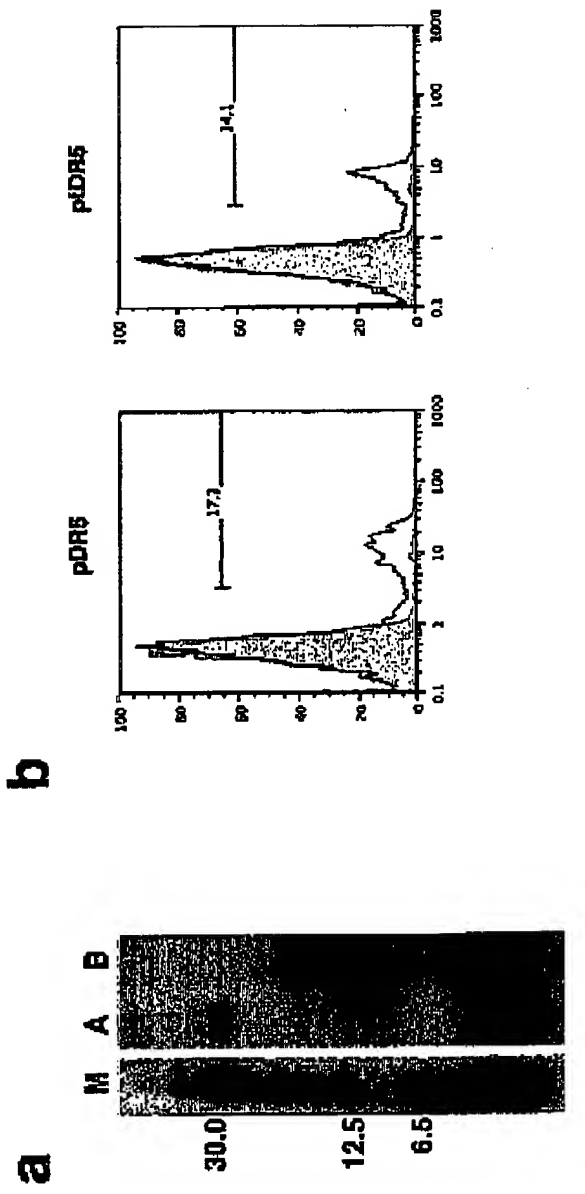

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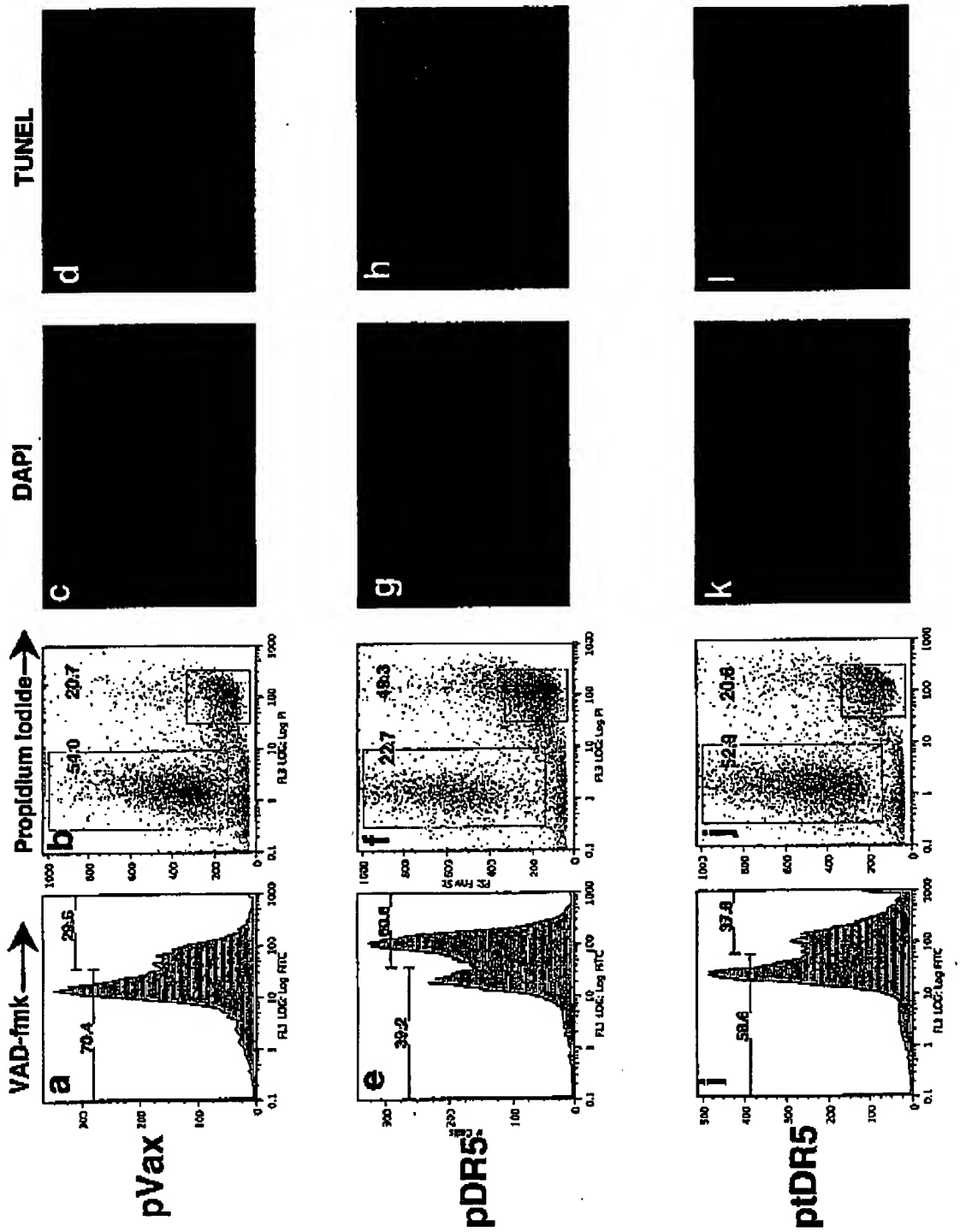

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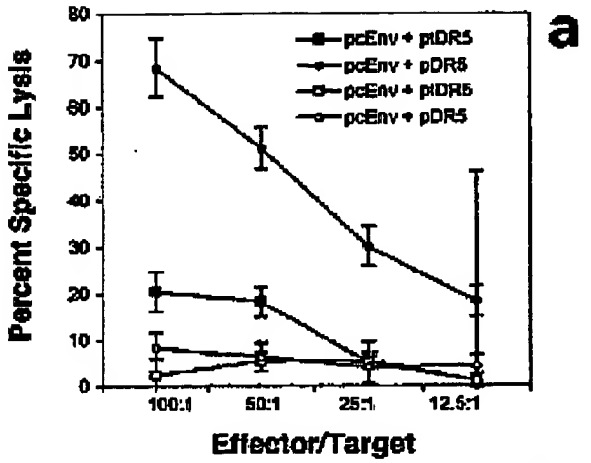

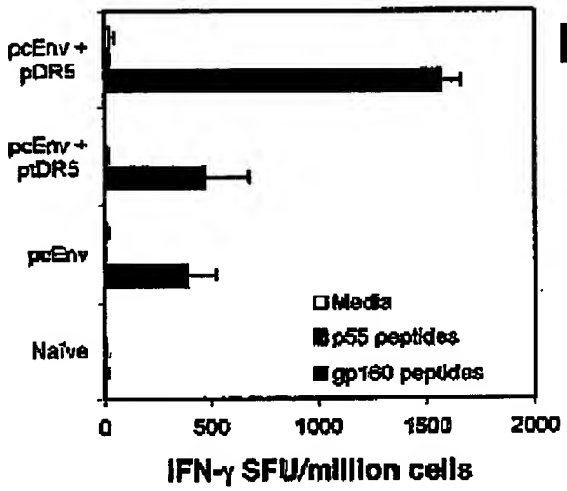

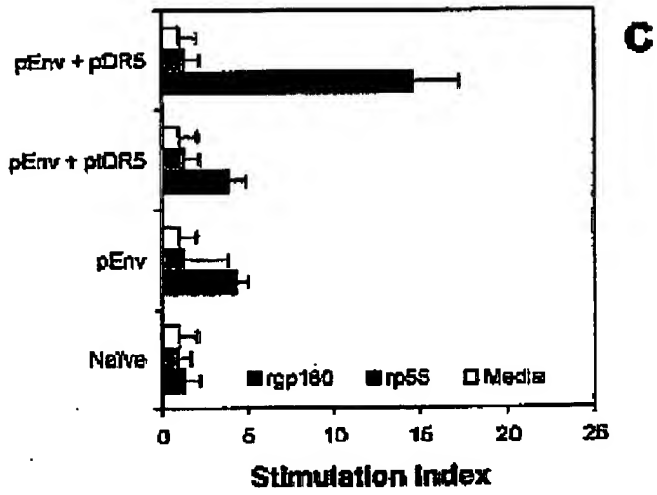

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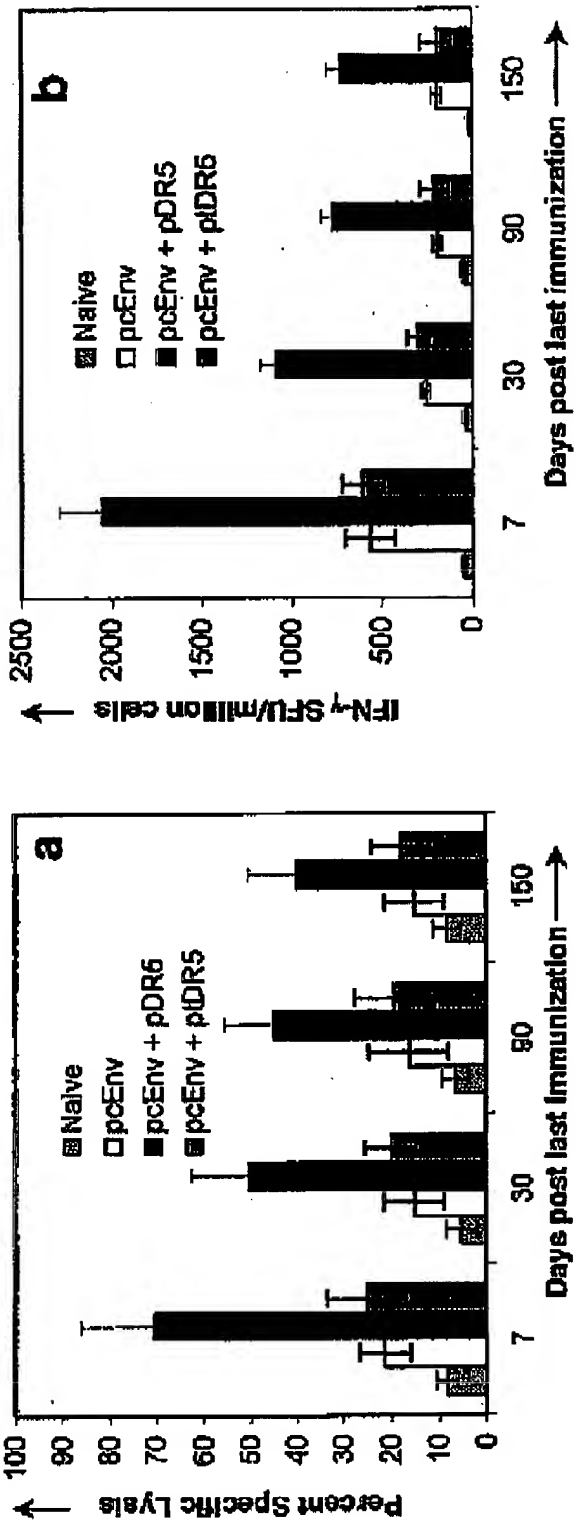
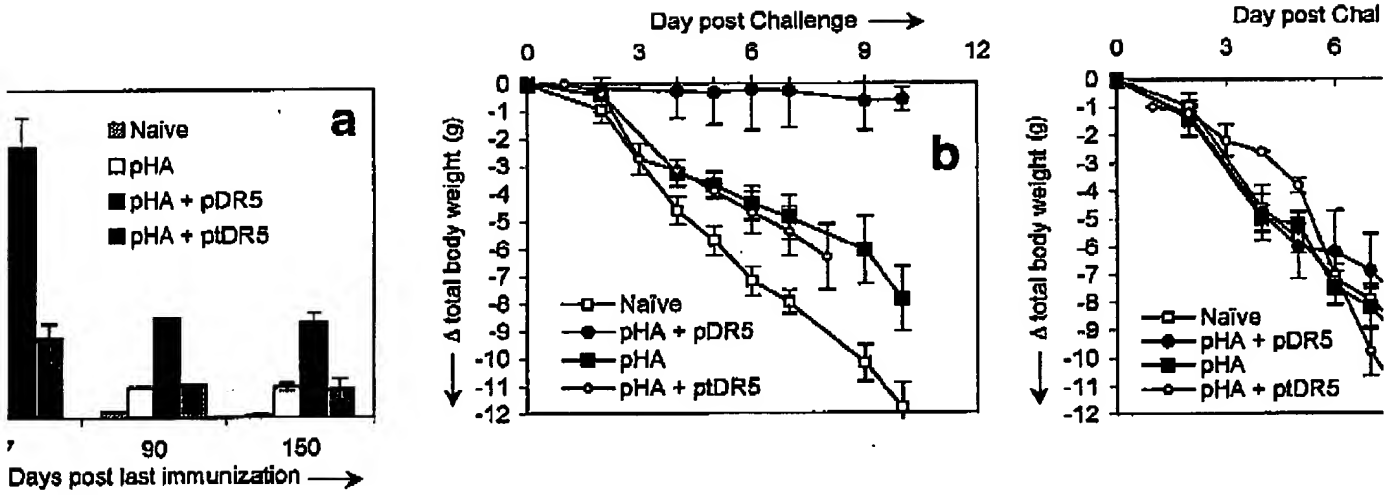


Chattergoon et. al. Figure 6



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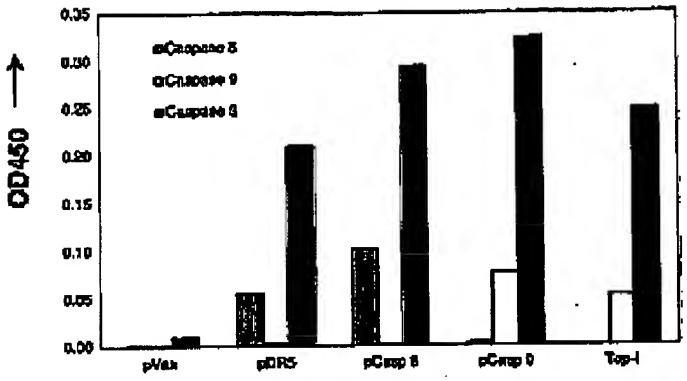

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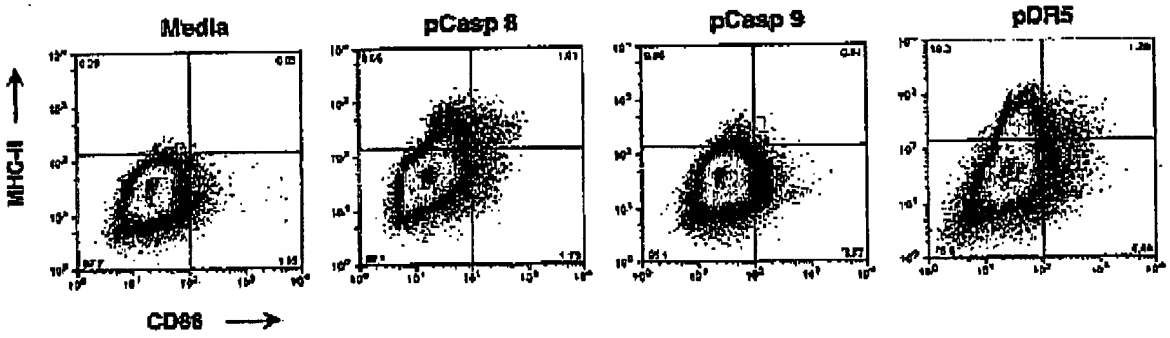

Chattergoon et. al. Figure 8